

Mutations Which Alter the Level or Structure of nsP4 Can Affect the Efficiency of Sindbis Virus Replication in a Host-Dependent Manner

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Two mutants of Sindbis virus have been isolated which grow inefficiently at 34.5°C in mosquito cells yet replicate normally in chicken embryo fibroblast cells at the same temperature. In addition, these mutants exhibit temperature-sensitive growth in both cell types and are RNA⁻ at the nonpermissive temperatures (K. J. Kowal and V. Stollar, *Virology* 114:140-148, 1981). To clarify the basis of this host restriction, we have mapped the causal mutations for these temperature-dependent, host-restricted mutants. Functional mapping and sequence analysis of the mutant cDNAs revealed several mutations which mapped to the amino terminus of nsP4, the putative polymerase subunit of the viral RNA replicase. These mutations resulted in the following amino acid changes in nsP4: leucine to valine at residue 48, aspartate to glycine at residue 142, and proline to arginine at residue 187. Virus containing any of these mutations was restricted in its ability to replicate in mosquito but not chicken embryo fibroblast cells at 34.5°C. In addition to its temperature-dependent, host-restricted phenotype, virus derived from one cDNA clone also exhibited decreased levels of nsP34 and nsP4 yet contained only a silent change in its genome. This C-to-U mutation occurred at nucleotide 5751, the first nucleotide after the opal termination codon separating nsP3 and nsP4. Our results suggest that this substitution decreases readthrough of the opal codon and diminishes production of nsP34 and nsP4. Such a decrease in synthesis rates might lead to levels of these products which are insufficient for viral RNA replication in mosquito cells at the higher temperature. This work provides the first evidence that nsP4 function can be strongly influenced by the host environment.

Alphaviruses are small, enveloped, plus-strand RNA viruses which are transmitted in nature by mosquitoes to their vertebrate hosts. Their continued propagation is dependent on their ability to replicate efficiently in both of these hosts, making them good models for studying the role of the host cell in viral replication (for a review, see reference 10). Comparisons of the replication of Sindbis virus, the prototype of the alphavirus genus, in vertebrate and invertebrate cells has revealed marked differences in Sindbis virus maturation and replication in the two cell types (for a review, see references 7 and 41). In addition to differences with respect to the site of virus maturation and ultrastructural changes in the infected host cell, the most noticeable contrast between infection of vertebrate and invertebrate cells with Sindbis virus concerns the effect of infection on the host cell. Replication of virus in vertebrate cells results in the shutdown of host protein synthesis and subsequent cell death, whereas in mosquito cells, virus can, depending on the host cell and the specific conditions, replicate to comparable levels in the absence of any visible cytopathic effect. These differences in host cell response to viral infection emphasize the importance of the host environment in determining the outcome of infection.

Replication of Sindbis virus is thought to require the activities of four nonstructural proteins which are translated

from the genomic 49S RNA as two polyprotein precursors which are cleaved to produce the four nonstructural proteins, nsP1 to nsP4 (46). The first polyprotein terminates at an opal termination codon and encodes nsP1, nsP2, and nsP3. The second polyprotein encodes all four nonstructural proteins, producing nsP4 by readthrough of the opal termination codon (44-46). The four nonstructural proteins (or a subset of them) are thought to form the viral replicase-transcriptase complex that initiates viral replication by synthesizing a full-length minus strand complementary to the genomic plus-strand RNA. Although viral replication requires host macromolecules and translational machinery common to all eucaryotic cells, studies with alphaviruses also suggest the involvement of additional host-specific components in both vertebrate (1, 3, 4) and invertebrate (11, 16, 40) cell cultures. Two approaches have been used to determine which steps in the virus life cycle may require host components. One method involves perturbing the host with transcription or translation inhibitors and examining the effects on viral replication. Using this approach, studies by Baric et al. (3, 4) suggest that replication of Sindbis virus in vertebrate cells is dependent on the involvement of a dactinomycin- and α -amanitin-sensitive host component(s) that is necessary for the synthesis of negative-strand RNA. In invertebrate cells, a host factor is required for maturation of Sindbis virus (40), and a dactinomycin-sensitive host component may be involved in the early stages of replication in order to produce viral RNA (11). This appears to be a labile host component whose continued synthesis is necessary for viral RNA replication to occur.

The second approach to identifying replicative events

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requiring host factors involves the generation of viral mutants that exhibit host-dependent phenotypes. This method allows the assignment of mutations to specific viral proteins, yielding information on which protein(s) may interact with host components to regulate virus replication. Following chemical mutagenesis, two such mutants of Sindbis virus were isolated and characterized (27). These mutants, called clones 35 and 58, replicate efficiently in mosquito cells at 28 but not 34.5°C. They are also temperature sensitive in chicken embryo fibroblast (CEF) cells, growing efficiently at 34.5 but not 40°C. Although the temperature ranges over which CEF and mosquito cells can grow in vitro are quite different, both cell types can grow and be maintained at 34.5°C, a temperature at which standard Sindbis virus can replicate efficiently in the two cell types. The fact that clones 35 and 58 replicate efficiently in mosquito cells at 28°C and in CEF cells at 34.5°C yet not in mosquito cells at 34.5°C indicates that their replication is host restricted in a temperature-dependent manner. In this report, we have used a cDNA clone of Sindbis virus (33) to map the changes responsible for this phenotype.

MATERIALS AND METHODS

Cell culture. Cultures of CEF cells were propagated as previously described (42). Second-passage cells were used for all experiments. The *Aedes albopictus* C7-10 cell line, derived from the LT-C7 clone, was maintained at 30°C in DME HG/HB (Dulbecco modified medium containing high glucose-high bicarbonate [44 mM]) containing nonessential amino acids and 10% fetal bovine serum (FBS). This cell line exhibits a marked cytopathic effect after infection with Sindbis virus at 34.5°C (37). Low-passage C7-10 cells were used for all experiments.

Virus stocks and plaque assay. To generate first-passage virus stocks, RNA transcripts of a full-length Sindbis virus cDNA clone (referred to as Toto1101) or the recombinant cDNA clones were synthesized in vitro by using SP6 RNA polymerase and were used to transfect secondary CEF monolayers as previously described (33). The transfection mix was removed and replaced with Eagle minimal essential medium with Earle salts (MEM) containing 2% FBS. After incubation at 30°C for 48 h, the culture supernatants were harvested and stored at -80°C. Sindbis virus derived from the cDNA clone Toto1101 will be referred to as Toto1101 virus.

Plaque assays were performed as previously described on CEF (42) or C7-10 (27) monolayers, with minor modifications. Plaques were visualized by staining with either neutral red or crystal violet after 72 h of incubation at 34.5°C for C7-10 cells or 48 h at 34.5°C and 40°C for CEF cells.

To test directly for temperature-sensitive plaque formation in CEF cells, the transfection mix was removed and the cells were overlaid with 1.4% agarose in MEM containing 2% FBS. Plaques were visualized by crystal violet staining after incubation at 34.5 or 40°C for 48 h.

General recombinant DNA techniques. Plasmids were grown and purified by the alkali lysis method with minor modifications (31). Restriction endonucleases and DNA-modifying enzymes were purchased from commercial sources and used as recommended by the manufacturers.

cDNA cloning and rescue of mutant phenotypes. Clone 35 and clone 58 virus stocks (27) were plaque purified and amplified on CEF monolayers at 30°C. Cultures of CEF cells in roller bottles were infected with virus at 30°C; at 36 h postinfection (p.i.), viral RNA was isolated and used for

cDNA synthesis as previously described (45). A subregion of the double-stranded cDNA from clone 58 was isolated by digestion with *Bgl*III (nucleotide [nt] 2288; nucleotide positions are given relative to the 5' end of the Sindbis virus 49S RNA [45]) and *Aar*II (nt 7999) and cloned into the homologous region of the plasmid Toto1101, which contains a full-length cDNA copy of the Sindbis virus genome (33). For clone 35, a subregion of the double-stranded cDNA was isolated by digestion with *Clal* (nt 2713) and *Aar*II (nt 7999) and cloned into the homologous region of the plasmid Toto1101. These recombinant plasmids were used as templates for the production of capped RNA transcripts which were infectious when used to transfect CEF monolayers (33). cDNA clones that gave rise to mutant phenotypes similar to those of clones 35 and 58 were selected for further analysis. Two cDNA clones derived from virus clone 58 (termed 58.1 and 58.2) and one from virus clone 35 (termed 35.1) were examined in detail.

Mapping of causal mutations. Subcloning was performed by replacing regions of Toto1101 with the corresponding regions of the mutant cDNAs. Mutant cDNA segments were inserted into Toto1101 deletion vectors to facilitate the selection of correct subclones. Gross mapping was first performed by dividing the mutant cDNAs into two subregions, one from *Clal* (nt 2713) to *Spe*I (nt 5262) and the other from *Spe*I (nt 5262) to *Aar*II (nt 7999). These restriction fragments were ligated into Toto1101 deletion vectors TPv1 and T23, respectively. TPv1 contained a 2,057-nt deletion from nt 3103 to 5160 created by removal of a *Pvu*II fragment. T23 was made by digesting Toto1101 with *Kpn*I (nt 5294) and *Hpa*I (nt 6919), producing blunt ends with T4 DNA polymerase, and religating to yield a 995-nt deletion.

Genomes containing overlapping segments of the region of interest were constructed for fine mapping. Since appropriate unique restriction sites were not present in the Toto1101 plasmid derivatives, fragments were subcloned into an intermediate plasmid which contained the necessary unique restriction sites. This plasmid, π nsP34C, consisted of the *Pvu*II (nt 5160)-to-*Nco*I (nt 8038) region of Toto1101 subcloned into π an9 which had been digested with *Hind*III and *Eco*RI and then filled in with Klenow fragment. π an9 was made by inactivating the *Dde*I site of π an8 by filling in with Klenow fragment (S. A. Chervitz, unpublished data). π an8, a derivative of π an7, has an *Sst*I linker (5'-CGAGCTCG3') inserted into the *Hinc*II site of π an7 (30). To eliminate wild-type background during subcloning, three different deletions were made in π nsP34C, creating vectors 1.8B, 2.12, and 3.2. Vector 1.8B was made by digesting π nsP34C with *Kpn*I (nt 5960) and *Pst*I (nt 5824), producing blunt ends with T4 DNA polymerase, and religating to yield a 136-nt deletion. Vector 2.12 was created by digesting π nsP34C with *Kpn*I (nt 5924) and *Eco*RV (nt 6878), producing blunt ends with T4 DNA polymerase, and religating to yield a 954-nt deletion. Vector 3.2 contained a 153-nt deletion from nt 6879 to nt 7031 created by removal of a *Dra*I (nt 7031)-to-*Eco*RV (nt 6878) fragment with subsequent ligation. These deletion vectors were used to subclone three overlapping regions of the mutant cDNAs: *Spe*I (nt 5262) to *Hind*III (nt 6267), *Pst*I (nt 5824) to *Hpa*I (nt 6919), and *Nsi*I (nt 6461) to *Bam*HI (nt 7334). Clones containing the substituted mutant cDNAs were identified by restriction analysis, and the *Spe*I (nt 5262)-to-*Aar*II (nt 7999) fragment from these constructs was used to replace the corresponding region in T23, thereby regenerating full-length recombinant Sindbis virus cDNA clones containing specific regions derived from the mutant cDNA.

Additional subcloning to separate clustered mutations was performed with the π nsP34C deletion vectors. An *SpeI* (nt 5262)-to-*PstI* (nt 5824) fragment was subcloned from 58.1, and a *HindIII* (nt 6267)-to-*AatII* (nt 7999) fragment was subcloned from 35.1. The *SpeI* (nt 5262)-to-*AatII* (nt 7999) region from these constructs was used to replace the corresponding region in T23.

Nucleotide sequence analysis. The DNA sequence of the entire region between the *SpeI* (nt 5262) and *HpaI* (nt 6919) sites was determined for clones 35.1, 58.1, and 58.2 by the dideoxy-chain termination method (36). Virion RNA isolated from the original virus clones 35 and 58 was sequenced directly (49) to confirm the presence of any mutations.

Sequence analysis of 58.1 revertants. CEF monolayers were infected with clone 58.1C1, overlaid with 1.4% agarose in MEM containing 2% FBS, and incubated at 34.5°C for 48 h. After being stained with neutral red, virus was isolated from small and medium plaques and used to generate virus stocks at 30°C on CEF monolayers. Total cytoplasmic RNA was isolated from dactinomycin-treated CEF cells infected with these stocks (48) and used for cDNA synthesis with avian myeloblastosis virus reverse transcriptase as previously described (18). One picomole of the negative-sense primer 5'-AATTTCTTTTAGTTGCGGCCAA3' (called PCR₂ and complementary to the Sindbis virus 49S RNA from nt 6364 to 6384) was used for cDNA synthesis. The DNA product was then amplified by the polymerase chain reaction (35) by asymmetric amplification (19). The reaction mixture (18), including 50 pmol of the positive-sense primer 5'-AAGTACATAGAAGTGCCA3' (called PCR₁ and corresponding to nt 5070 to 5087 of the Sindbis virus 49S RNA), 1 pmol of PCR₂, and 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus), was subjected to 30 reaction cycles (1 min at 92°C, 1 min at 45°C, and 3.5 min at 72°C). The polymerase chain reaction products were extracted with chloroform followed by phenol and then chloroform, and the DNA was collected by ethanol precipitation and sequenced by the dideoxy method (36).

RNA shift up. CEF monolayers in 12-well tissue culture plates were infected at a multiplicity of 20 PFU per cell with virus diluted in phosphate-buffered saline containing 1% FBS and 1 µg of dactinomycin per ml (all samples were done in triplicate). After 1 h of adsorption at 4°C, the inoculum was replaced with prewarmed MEM containing 2% FBS and 1 µg of dactinomycin per ml, and the cells were incubated at 30, 34.5, or 40°C. Viral RNA was labeled from 3 to 6 h p.i. in the presence of MEM containing 2% FBS, 1 µg of dactinomycin per ml, and 20 µCi of [³H]uridine per ml. At the start of labeling, cells were either kept at their original incubation temperatures or shifted from 30 and 34.5 to 40°C. At 6 h p.i., cells were washed three times with ice-cold phosphate-buffered saline and then lysed with 200 µl of lysis solution (0.5% Triton X-100, 10 mM Tris chloride [pH 7.5], 1 mM EDTA, 100 mM NaCl). Relative levels of RNA synthesis were determined by measuring incorporation by trichloroacetic acid precipitation. The sample (25 µl) was spotted onto 3-mm disks (Whatman, Inc.), and the disks were dried and fixed for 20 min in 20% trichloroacetic acid. The disks were washed two times for 20 min each time with 5% trichloroacetic acid, once for 5 min in ethanol:ether (3:1), and once for 5 min in ether. The disks were dried and counted in toluene scintillation fluid.

C7-10 monolayers in 12-well tissue culture plates were infected at a multiplicity of 100 PFU per cell (the virus titer was determined on CEF cells) with virus diluted in phosphate-buffered saline containing 1% FBS (all samples were

done in triplicate). After 1 h of adsorption at 4°C, the inoculum was replaced with prewarmed DME HG/HB containing 10% FBS plus nonessential amino acids, and the cells were incubated at 28 or 34.5°C. At 30 min prior to the labeling of viral RNA, the medium was replaced with prewarmed DME HG/HB containing 10% FBS, nonessential amino acids, and 1 µg of dactinomycin per ml. Viral RNA was labeled from 22 to 25 h p.i. with DME HG/HB containing 10% FBS, nonessential amino acids, 1 µg of dactinomycin per ml, and 20 µCi of [³H]uridine per ml. At the start of labeling, cells were either kept at their original incubation temperatures or shifted from 28 to 34.5°C. At the end of the labeling period, cells were lysed and relative levels of RNA synthesis were determined as described above.

Complementation. Complementation was performed as described by Burge and Pfefferkorn (9), with minor modifications. CEF monolayers in 12-well tissue culture plates were infected at a multiplicity of 50 PFU per cell with either two mutants (each at 50 PFU per cell) or each mutant alone (at 50 PFU per cell) for 1 h at 40°C. Cells were washed with medium at 40°C and then incubated at 40°C in prewarmed medium containing 1 µg of dactinomycin per ml. After 2 h, the medium was replaced with fresh medium without dactinomycin. At 6 h p.i., the supernatant was collected and stored at -80°C. The titer was later determined by plaque assay on CEF monolayers at 28°C.

Analysis of viral nonstructural proteins. CEF monolayers in 35-mm dishes were infected for 1 h at 4°C at a multiplicity of infection of 20 PFU per cell. Cells were washed with prewarmed MEM and incubated at 34.5°C in MEM containing 2% FBS. At 3 h p.i., the medium was removed and replaced with prewarmed MEM lacking methionine. At 3.5 h p.i., the medium was replaced with prewarmed methionine-free MEM which contained 2% FBS and 20 µCi of [³⁵S]methionine per ml, and the cells were labeled for 1 h at 34.5°C. The cells were lysed as previously described (29), and the lysates were used for immunoprecipitation (21). Immunoprecipitates were heated at 95°C for 3 min and then separated by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis. Gels were fluorographed (28), dried, and exposed to X-ray film.

RESULTS

Sindbis virus derived from Toto1101 was able to produce plaques on both CEF and *A. albopictus* cells at 34.5°C, although the titer on C7-10 mosquito cells was approximately 12-fold lower than that obtained on CEF cells and plaques took about 24 h longer to appear. This virus was not temperature sensitive in CEF cells, producing plaque almost as efficiently at 40°C in these cells as at 34.5°C. These observations indicated that the Toto1101 genetic background would be appropriate for identifying the mutation(s) responsible for the phenotypes of clones 35 and 58. The observation that at the nonpermissive temperature the mutants were RNA⁻ but showed no defect in adsorption or penetration (27) suggested that the mutations responsible for the host-restricted phenotype were in the genes encoding the viral nonstructural proteins. In addition, these mutants belong to the F complementation group, and the causal lesions for several members of this group have recently been mapped to nsP4 (20). We therefore decided to examine the region of the Sindbis virus genome encoding nsP3 and nsP4, and cDNAs of clones 35 and 58 were made which encompassed this region.

Construction of recombinant Sindbis virus genomes. Viral

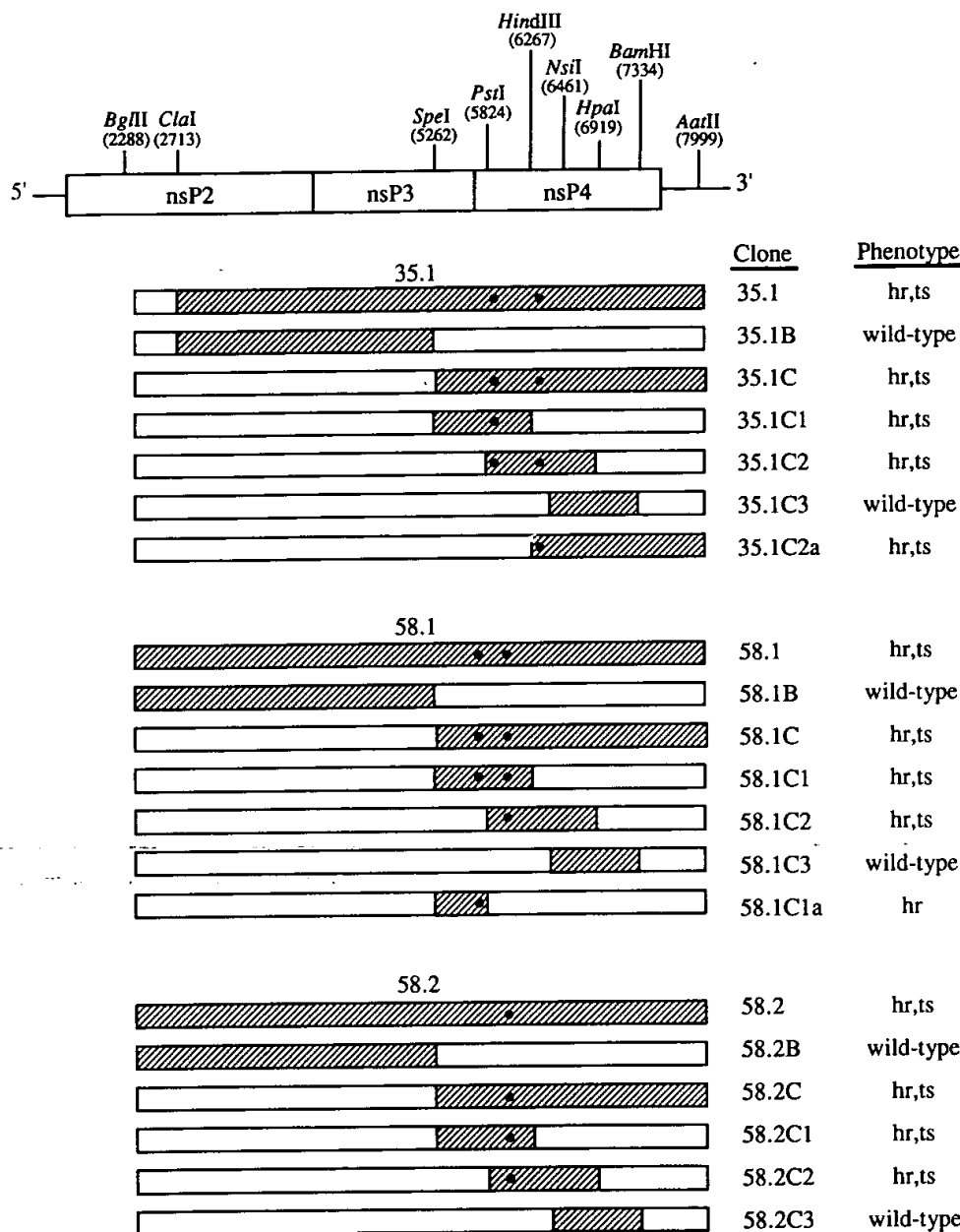


FIG. 1. Construction of hybrid genomes. A schematic of the protein-coding region for the Sindbis virus nonstructural proteins nsP3, nsP4, and part of nsP2 is shown along with the restriction sites used for subcloning into Sindbis virus cDNA clone Toto1101 (33). Symbols: ▨, regions of Toto1101 which were replaced with the corresponding regions from the mutant cDNAs; ●, sites at which mutations relative to Toto1101 were found in the genome. hr, Host restricted for mosquito cells; ts, temperature sensitive in CEF cells.

RNA was isolated from mutants 35 and 58 and used to construct recombinant Sindbis virus cDNA clones with mutant cDNA from *Bgl*II (nt 2288) to *Aat*II (nt 7999) for clone 58 and from *Cla*I (nt 2713) to *Aat*II (nt 7999) for clone 35 (Fig. 1). Three cDNA clones were examined in detail, two of clone 58 (58.1 and 58.2) and one of clone 35 (35.1). Virus stocks were generated from the recombinant plasmids and tested for host restriction by comparing plaquing efficiencies on mosquito cells versus CEF cells at 34.5°C (Table 1). In addition, temperature sensitivity in CEF cells was assayed by comparing plaquing efficiencies on CEF cells at 34.5

versus 40°C (Table 1). Temperature sensitivity of plaque formation in *A. albopictus* cells could not be tested directly by comparing plating efficiencies at 34.5 versus 28°C because the C7-10 cells did not give rise to plaques at 28°C. Consequently, temperature sensitivity in mosquito cells was examined by comparing virus yields at 28 and 34.5°C (Table 2). The original viral clones 35 and 58 were included in all experiments for comparison with virus derived from cDNA clones.

Viruses derived from cDNA clones 35.1 and 58.2 were host restricted in C7-10 cells and temperature sensitive in

TABLE 1. Plating efficiency of Sindbis virus mutants on CEF and *A. albopictus* cells^a

Clone	Titer (PFU/ml) when assayed on:			Efficiency of plating	
	CEF at 40°C	CEF at 34.5°C	C7-10 at 34.5°C	C7-10/CEF (34.5°C)	CEF (40°C)/CEF (34.5°C)
Toto1101	5.0×10^8	6.0×10^8	5.0×10^7	8.3×10^{-2}	8.3×10^{-1}
35	1.8×10^5	9.0×10^6	$<5.0 \times 10^1$	$<5.5 \times 10^{-6}$	1.9×10^{-2}
35.1	5.0×10^5	1.0×10^7	$<5.0 \times 10^1$	$<5.0 \times 10^{-6}$	5.0×10^{-2}
35.1C1	4.0×10^7	5.0×10^8	1.0×10^4	2.0×10^{-5}	8.0×10^{-2}
35.1C2	1.0×10^7	3.0×10^8	$<5.0 \times 10^1$	$<1.0 \times 10^{-7}$	3.0×10^{-2}
35.1C3	1.0×10^7	5.0×10^7	1.0×10^6	2.0×10^{-2}	2.0×10^{-1}
35.1C2a	4.0×10^7	5.0×10^8	5.0×10^4	1.0×10^{-4}	8.0×10^{-2}
58	2.0×10^4	4.0×10^8	$<5.0 \times 10^2$	$<1.3 \times 10^{-6}$	5.0×10^{-5}
58.1	$<5.0 \times 10^1$	2.0×10^{8b}	$<5.0 \times 10^1$	$<2.0 \times 10^{-7}$	$<2.0 \times 10^{-7}$
58.1C1	$<5.0 \times 10^1$	3.5×10^{8b}	$<5.0 \times 10^1$	$<1.0 \times 10^{-7}$	$<1.0 \times 10^{-7}$
58.1C2	5.0×10^3	7.0×10^8	$<5.0 \times 10^2$	$<7.0 \times 10^{-7}$	7.0×10^{-6}
58.1C3	2.0×10^9	3.0×10^9	3.0×10^7	1.0×10^{-2}	6.7×10^{-1}
58.1C1a	1.0×10^8	2.5×10^8	7.0×10^2	2.8×10^{-6}	4.0×10^{-1}
58.2	1.0×10^5	2.0×10^9	$<5.0 \times 10^2$	$<2.5 \times 10^{-7}$	5.0×10^{-5}
58.2C1	1.0×10^4	1.0×10^9	$<5.0 \times 10^2$	$<5.0 \times 10^{-7}$	1.0×10^{-5}
58.2C2	5.0×10^3	5.0×10^8	$<5.0 \times 10^2$	$<1.0 \times 10^{-6}$	1.0×10^{-5}
58.2C3	7.0×10^8	2.0×10^9	2.5×10^7	1.3×10^{-2}	3.5×10^{-1}

^a Titers of virus stocks were determined by plaque assay (see Materials and Methods).^b Plaques were a heterogeneous population (see Results).

CEF cells, as were viral clones 35 and 58 (Tables 1 and 2). Virus derived from cDNA clone 58.1 was also host restricted but exhibited increased temperature sensitivity in CEF cells as compared with that exhibited by the original mutant, clone 58. Shorter segments of the Toto1101 infectious cDNA clone were then replaced with corresponding regions of the mutant cDNAs in order to localize the mutations (Fig. 1). Gross mapping showed that the *SpeI* (nt 5262)-to-*AatII* (nt 7999) fragment retained the mutant phenotype for all three clones. To further localize the mutations, subclones of this region were constructed (Fig. 1) and their phenotypes were assayed (Table 1).

Localization of causal mutations in clone 35.1. Assays of the initial subclones 35.1C1, 35.1C2, and 35.1C3 mapped the causal mutations to the region between the *SpeI* (nt 5262) and *HpaI* (nt 6919) sites (Fig. 1 and Table 1). DNA sequence

analysis of these clones revealed two mutations in the genome, C-to-G transversions at nt 5910 and nt 6328. Both mutations occurred in the region encoding nsP4 and changed the amino acid coding sequence from a Leu to a Val (amino acid 48 of nsP4) and from a Pro to an Arg (amino acid 187 of nsP4), respectively (Fig. 2). RNA sequencing of clone 35 virion RNA confirmed that both mutations were present in the original mutant. To determine the contribution of each mutation to the phenotype, further subcloning was performed to produce clone 35.1C2a, which contained only the mutation at nt 6328 (Fig. 1). The change at nt 5910 was already isolated in clone 35.1C1.

Virus with both mutations (35.1C2) was unable to produce plaques on mosquito cells at 34.5°C yet at the same temperature formed plaques on CEF cells as efficiently as did Toto1101 virus (Table 1). A similar phenotype was observed

TABLE 2. Replication of host-restricted mutants of Sindbis virus in CEF and *A. albopictus* cells at different temperatures^a

Clone	Titer (PFU/ml) of infectious virus released from:							
	C7-10 cells at:				CEF cells at:			
	28°C		34.5°C		34.5°C		40°C	
	4 h	28 h	4 h	28 h	1 h	6 h	1 h	6 h
Toto1101	1.6×10^4	8.5×10^6	1.0×10^4	1.9×10^7	9.0×10^4	4.8×10^8	6.3×10^4	2.7×10^8
35	2.7×10^4	1.1×10^7	3.5×10^4	1.1×10^4	3.4×10^4	4.0×10^7	5.5×10^4	2.6×10^6
35.1C1	1.8×10^4	2.3×10^7	2.3×10^4	2.4×10^4	3.8×10^4	1.4×10^8	3.0×10^4	1.5×10^6
35.1C2	2.8×10^4	1.6×10^7	2.4×10^4	3.0×10^4	1.4×10^5	1.4×10^8	1.2×10^5	8.5×10^5
35.1C2a	1.5×10^4	1.2×10^7	1.2×10^4	1.3×10^6	6.5×10^4	9.0×10^7	4.6×10^4	1.5×10^6
58	1.8×10^4	1.4×10^8	2.2×10^4	1.7×10^5	6.5×10^4	1.3×10^8	3.7×10^4	7.5×10^3
58.1C1	2.2×10^4	3.2×10^7	2.0×10^4	2.8×10^3	8.0×10^3	1.1×10^8	1.0×10^4	6.0×10^3
58.1C2	1.5×10^4	2.8×10^7	2.0×10^4	1.5×10^3	6.7×10^4	1.1×10^8	6.0×10^4	3.3×10^5
58.1C1a	2.0×10^4	3.5×10^6	1.9×10^4	2.8×10^4	1.0×10^5	8.5×10^7	1.2×10^5	4.7×10^7
58.2C2	3.7×10^4	3.1×10^7	3.2×10^4	2.2×10^3	6.9×10^4	1.1×10^8	8.3×10^4	2.6×10^5

^a CEF or mosquito cell monolayers were infected with an input multiplicity of 10 PFU per cell. Adsorption was for 1 h at 4°C. Cells were washed three times with phosphate-buffered saline, prewarmed medium was added, and the cultures were incubated at the indicated temperatures. Virus samples were collected at the indicated times p.i., and titers were determined by plaque assay on CEF monolayers at 28°C. Shown here are the time points at which maximum titers could be obtained at the higher temperature for each cell type.

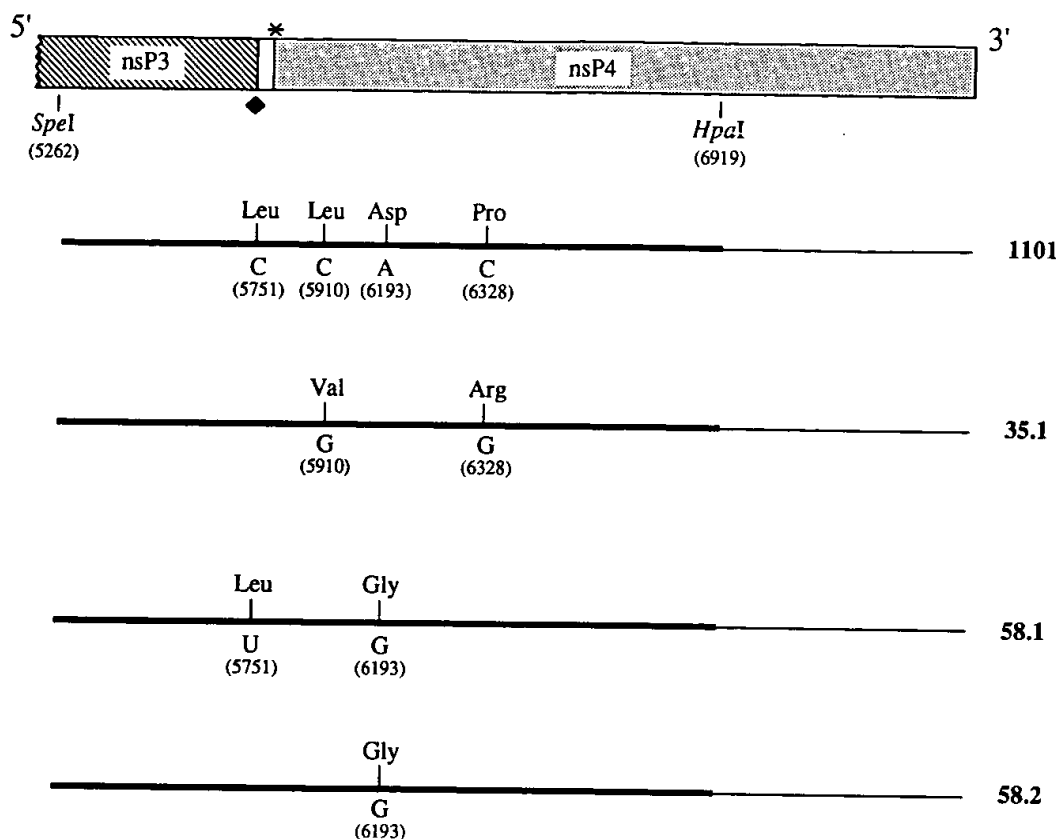


FIG. 2. Causal mutations in 35.1, 58.1, and 58.2 cDNA clones. A schematic of nsP4 and the surrounding regions of the Sindbis virus genome is shown. Below are shown schematics of the changes found in cDNA clones 35.1, 58.1, and 58.2. Symbols: *, the putative N terminus of nsP4; ◆, the opal termination codon preceding nsP4; —, regions sequenced. Nucleotide and amino acid changes in the mutant cDNAs are indicated. Nucleotide positions where changes occurred are shown below the line, and the resulting amino acids are shown above the line.

for viral clone 35 and virus generated from cDNA clone 35.1. Virus stocks containing the separated mutations (35.1C1 and 35.1C2a) had plating efficiencies on C7-10 cells 100- to 1,000-fold lower than that for Toto1101 virus (Table 1), indicating that each mutation alone decreased the ability of virus to produce plaques on mosquito cells.

Although clone 35 had been previously reported to be temperature sensitive in CEF cells (27), we observed only a slight temperature sensitivity (~10-fold) both for virus derived from cDNA clones and for the original viral clone 35. The earlier finding that a revertant which was no longer temperature sensitive was also no longer host restricted suggested that the same lesion was responsible for both the temperature-sensitive and host-dependent phenotypes (27). This has been difficult to confirm in the present experiments because the temperature-sensitive phenotype which we observed for clone 35 and its derivatives was less pronounced than what was originally observed.

We next studied the replication efficiencies of these mutants by comparing virus growth in mosquito cells at 28 and 34.5°C and in CEF cells at 34.5 and 40°C. Toto1101 virus replicated efficiently in both cell types at the temperatures tested (Table 2). Virus containing both mutations (35.1C2) or the single mutation at nt 5910 (35.1C1) replicated as efficiently as Toto1101 virus in CEF cells at 34.5°C yet replicated 10³-fold less efficiently than Toto1101 in mosquito cells at the same temperature. In addition, these viruses were temperature sensitive in mosquito cells, exhibiting 10³-fold

more efficient replication at 28°C than at 34.5°C, yet growth in CEF cells was only moderately temperature sensitive. Virus with the single mutation at nt 6328 (35.1C2a) was not host restricted but was slightly temperature sensitive in both mosquito and CEF cells.

Localization of causal mutations in clones 58.1 and 58.2. Mutations responsible for the host-restricted phenotypes of 58.1 and 58.2 also mapped within the *SpeI* (nt 5262)-to-*HpaI* (nt 6919) region (Fig. 1 and Table 1). Sequencing of the cDNAs showed that both clones contained an A-to-G transition at nt 6193, resulting in an Asp-to-Gly change at position 142 of nsP4 (Fig. 2). Virus containing only this mutation (58.1C2) had a plating efficiency on C7-10 cells at 34.5°C 10⁵-fold lower than that for wild-type virus (Table 1). In addition, the virus was temperature sensitive for plaque formation in CEF cells, with a 10⁵-fold reduction in titer on CEF cells at 40°C as compared with that at 34.5°C. Although the virus replicated as efficiently as Toto1101 virus in CEF cells at 34.5°C, it did not replicate in mosquito cells at the same temperature and exhibited temperature-sensitive growth in both CEF and mosquito cells. The phenotype of this virus is similar to that seen for viral clone 58 (Tables 1 and 2).

Although similar to clone 58.2 in its host restriction, virus derived from cDNA clone 58.1 exhibited increased temperature sensitivity in CEF cells. This virus did not produce plaques on CEF cells at 40°C and gave rise to a population of small and medium plaques when plaqued on these cells at

TABLE 3. Complementation analysis of clones 58.1 and 58.2

Clone	Complementation index ^a			
	Group A (<i>ts17</i>)	Group B (<i>ts11</i>)	Group F (<i>ts6</i>)	Group G (<i>ts18</i>)
58	61	31	0.4	50
58.1C1	27	26	1.1	54
58.1C2	70	39	0.9	217

^a The yield of virus in a mixed infection at 40°C divided by the sum of the yields of infectious virus from CEF cultures infected with each mutant alone at 40°C.

34.5°C (Table 1). In addition to the change at nt 6193, clone 58.1 also had a silent C-to-U transition at nt 5751 which was not present in clone 58.2 (Fig. 2). When both mutations were present, the virus (58.1C1) was not only host restricted but also grew poorly in CEF cells at 40°C (Table 2). Analysis of the heterogeneous plaque population showed that the small plaques retained both mutations, whereas the medium plaques had reverted to the wild-type sequence at nt 5751 yet still had the A-to-G mutation at nt 6193. Virus derived from the medium-sized plaques gave rise to medium-sized plaques on CEF cells at 34.5°C, while virus derived from the small plaques again gave rise to a population of small and medium plaques.

To determine whether the silent mutation by itself had any effect on virus replication, we isolated the mutation by further subcloning (Fig. 1). Virus generated from this clone (58.1C1a) had a plating efficiency approximately 10⁴-fold lower on C7-10 versus CEF cells at 34.5°C than wild-type virus did (Table 1). Its growth was severely restricted in mosquito cells at 34.5°C, yet the virus replicated as efficiently as Toto1101 virus in CEF cells at the same temperature (Table 2). Although temperature sensitive in mosquito cells, this virus was not temperature sensitive in CEF cells and gave rise to a homogeneous population of medium-sized plaques (relative to plaques produced by Toto1101) on CEF cells at 34.5°C (Tables 1 and 2).

RNA sequencing of the original clone 58 virion RNA showed the same A-to-G mutation at nt 6193 as was seen with the cDNA clones but did not show the change at nt 5751. This was expected, since virus containing both mutations (58.1C1) exhibited a phenotype similar to that of clone 58.1 while virus containing only the mutation at nt 6193 (58.1C2, 58.2C1, and 58.2C2) behaved like the original viral clone 58 (Table 1). It is possible that the mutation at nt 5751 arose as an artifact of cDNA cloning. Alternatively, RNAs containing both mutations may have represented only a small percentage of the original RNA population which was cloned and, therefore, were not present at high enough levels to be detected by RNA sequencing.

Clones 58.1 and 58.2 complemented members of all RNA⁻ complementation groups except F. Several temperature-sensitive mutants of Sindbis virus which are defective in RNA replication and belong to the F complementation group have been shown to contain defects in nsP4 (20). These mutants complement representative members of all RNA⁻ complementation groups except group F. To determine whether clones 58.1 and 58.2 were behaving like other group F mutants, we performed complementation analysis using temperature-sensitive mutants originally derived by Burge and Pfefferkorn (9) (Table 3). The subclones complemented representative members of all RNA⁻ complementation groups tested except for the group F mutants, which also contain defects in nsP4 (20). Containing only the mutation at

TABLE 4. Efficiency of viral RNA synthesis in CEF and *A. albopictus* cells

Clone	RNA synthesis ^a in:					
	CEF cells ^b at:			C7-10 cells at:		
	34.5°C	40°C	Shift up	28.0°C	34.5°C	Shift up
Toto1101	1.00	1.00	1.00	1.00	1.00	1.00
<i>ts6</i> ^c	0.06	0.02	0.03	0.56	0.00	0.22
35	0.96	0.27	0.74	0.55	0.10	0.39
35.1C1	1.00	0.41	0.85	1.06	0.23	0.93
35.1C2	0.85	0.20	0.73	0.63	0.08	0.47
35.1C2a	0.97	0.60	0.93	0.80	0.50	0.95
58	0.66	0.04	0.61	0.72	0.07	0.43
58.1C1	0.58	0.01	0.55	0.58	0.03	0.57
58.1C2	0.75	0.01	0.56	0.73	0.09	0.48
58.1C1a	0.68	0.33	0.55	0.95	0.16	0.60
58.2C1	0.83	0.02	0.73	0.86	0.07	0.54

^a Relative to Toto1101 virus, assayed by incorporation of trichloroacetic acid-precipitable material following infection at the indicated temperatures or after shift to the higher temperatures following infection at the lower temperatures as described in Materials and Methods.

^b Experiments were done at 30°C in parallel, to compare with levels of *ts6* RNA synthesis since *ts6* was already shut off at 34.5°C (data not shown).

^c Values for *ts6* in CEF cells are averages from two independent experiments.

nt 5751, 58.1C1a exhibited no complementation since it was not temperature sensitive in CEF cells. Because of their minimal temperature sensitivity in CEF cells, 35.1 clones could not be assayed.

RNA synthesis continued after shift up in both CEF and mosquito cells. Kowal and Stollar have shown that clones 35 and 58 are RNA⁻ at the nonpermissive temperatures (27). To determine whether RNA synthesis continues at the nonpermissive temperature once the infection had been established at the permissive temperature, we performed shift-up experiments with these mutants in both CEF and mosquito cells. *ts6*, a group F RNA⁻ temperature-sensitive mutant, was included as a control since it has been shown to shut off all RNA synthesis upon shift up to the nonpermissive temperature in CEF cells (24, 39). Incubation at 40°C in CEF cells showed that, as expected, all clone 58 mutants (apart from 58.1C1a) were RNA⁻ at the nonpermissive temperature (Table 4). However, shift up to the nonpermissive temperature after first establishing infection at the permissive temperature permitted significant levels of RNA synthesis. Although clone 35 mutants did not completely shut off RNA synthesis at 40°C, considerably more RNA was produced at 40°C when the infection was first established at the permissive temperature. Similar results were obtained with mosquito cells; RNA synthesis was strongly inhibited at the nonpermissive temperature but was able to continue at the nonpermissive temperature (Table 4) if first established at the permissive temperature. This indicates that in both cell types, the replication complex of these mutants remains stable upon shift up once it has already been formed at the permissive temperature. Similar results have been observed in CEF cells for another mutant of the F complementation group, *ts118* (20).

Context sequence affected readthrough efficiency of nsP4. Readthrough of termination codons can be influenced by the sequence context in which they occur (14); therefore, it was of interest to determine whether the silent change at nt 5751, which was immediately 3' to the UGA opal termination codon, influenced readthrough and production of nsP4 or

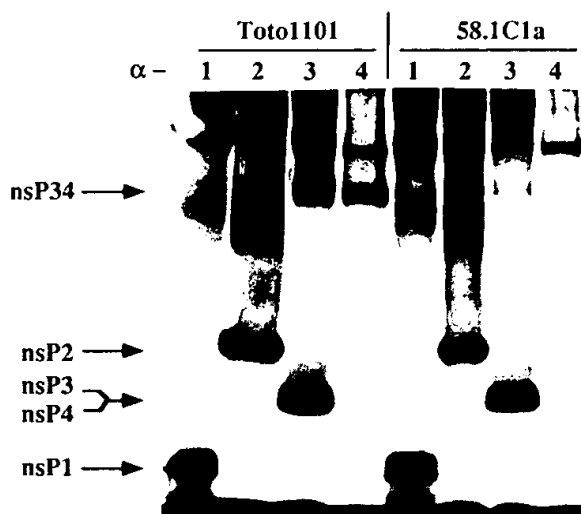


FIG. 3. Immunoprecipitation of viral nonstructural proteins. CEF monolayers were infected at a multiplicity of infection of 20 PFU per cell with either Toto1101 virus or mutant 58.1C1a. At 3.5 h p.i., the cells were labeled with [35 S]methionine for 1 h and then lysed with 1% SDS. Lysates were immunoprecipitated with Sindbis polyclonal antibodies monospecific for the individual nonstructural proteins and analyzed by SDS-8% polyacrylamide gel electrophoresis. Lanes 1, 2, 3, and 4, samples immunoprecipitated with Sindbis virus antibodies specific for nsP1, nsP2, nsP3, and nsP4, respectively. The positions of the Sindbis virus nonstructural proteins are indicated on the left; nonstructural proteins nsP3 and nsP4 comigrate.

nsP4. Immunoprecipitations with antibodies to the nonstructural proteins were performed on pulse-labeled cell lysates from CEF cells infected with virus generated from clone 58.1C1a or Toto1101. SDS-polyacrylamide gel electrophoretic analysis of the immunoprecipitations showed that virus containing the C-to-U change at nt 5751 produced lower levels of nsP4 and nsP34 than wild-type virus did, while levels of nsP3 were slightly elevated and those of nsP1 and nsP2 were unchanged (Fig. 3). Similar results were obtained with cell-free translation studies (G. Li, unpublished data). Thus, a C-to-U change at nt 5751 appears to specifically decrease production of nsP4 and nsP34. We were unable to do similar analyses of these proteins with infected mosquito cells because the levels of nsP34 and nsP4 were too low to be detected by immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

DISCUSSION

By mapping the causal mutations for two temperature-dependent, host-restricted mutants of Sindbis virus, we have obtained some insights into the interactions between the virus-specified replication machinery and the host environment. Two types of changes have been found which affect the virus nonstructural protein nsP4, those which map directly to nsP4 and one which affects the synthesis of nsP4. The mutations in nsP4 itself mapped to the amino-terminal domain of the protein and lay in regions of nsP4 which are highly conserved among alphaviruses (Fig. 4). The high degree of conservation of these amino acids, in addition to the fact that altering them renders a virus host restricted, indicates that these sites play an important role in the function of nsP4.

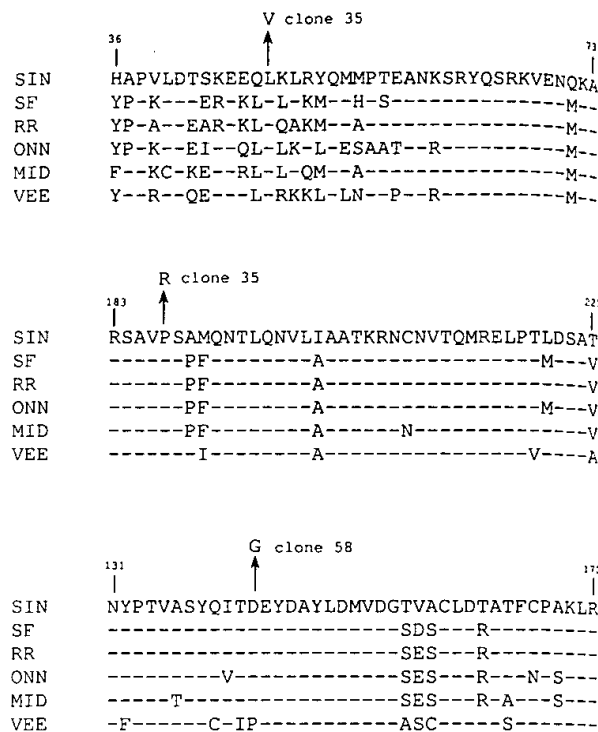


FIG. 4. Comparison of amino acid sequences from six alphaviruses in regions of nsP4 containing the causal changes for clones 35 and 58. Arrows indicate the amino acid which was altered. Sequence data are from the following sources: Sindbis virus (SIN), Strauss et al. (45); Semliki Forest virus (SF), Takkinen (47); Ross River virus (RR), Faragher et al. (17) and Strauss et al. (43); O'Nyong-nyong virus (ONN), Strauss et al. (43); Middelburg virus (MID), Strauss et al. (44); and Venezuelan equine encephalitis virus (VEE), Kinney et al. (25).

Although the four nonstructural proteins (or a subset of them) of Sindbis virus are thought to form the viral replicase-transcriptase complex that initiates viral replication, the role each protein plays in viral replication has not yet been resolved. nsP4 is postulated to function as the polymerase subunit of the viral RNA replicase (23), as it is highly conserved among alphaviruses and shares amino acid homology with the RNA-dependent RNA polymerase of poliovirus and with putative polymerase proteins of several plant and animal RNA viruses (2, 22, 23, 34). In addition, the causal lesion of *ts6*, a temperature-sensitive mutant defective in all RNA synthesis at the nonpermissive temperature, has recently been mapped to nsP4, further supporting the idea that nsP4 may function as the elongation subunit of the viral replicase (5, 8, 20, 24, 39). Whether the active form of the RNA polymerase is nsP4 itself or the nsP34 polypeptide is unknown. Recent genetic data also suggest a role for nsP4 in regulation of minus-strand synthesis (38).

Assuming nsP4 is the polymerase, it must not only contain the elongation function but probably also has domains which play a role in defining template specificity. These would include determinants for interaction with the other nonstructural proteins, initiation factors, and/or *cis*-acting RNA sequences to selectively initiate plus-strand, minus-strand, and subgenomic RNA synthesis. In addition, certain domains of nsP4 may interact either directly or indirectly with

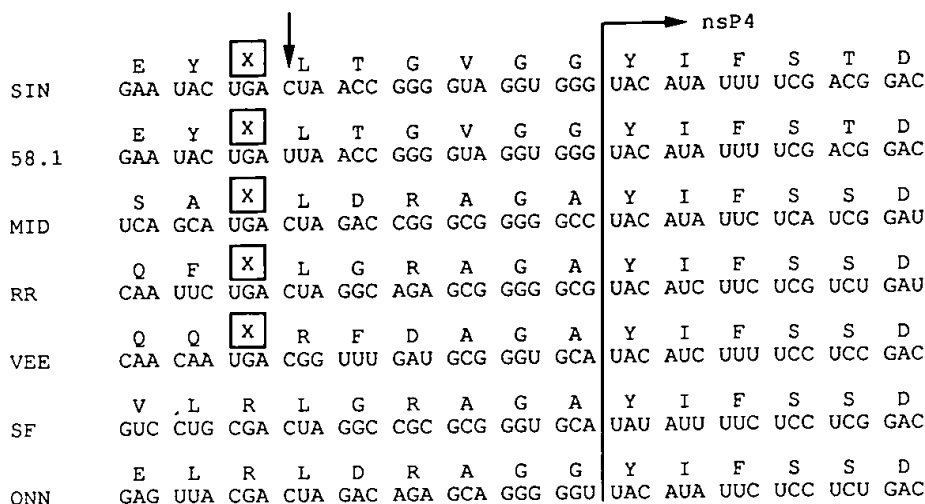


FIG. 5. Comparison of nucleotide sequences from six alphaviruses and clone 58.1 at the nsP3-nsP4 junction. The boxed X indicates the position of the opal codon when it occurs. The downward arrow indicates the cytosine residue at which the silent mutation occurs in clone 58.1. Shown also is the putative start site of nsP4 (45). Sequence data are from the sources given in the legend to Fig. 4. Abbreviations are defined in the legend to Fig. 4.

specific host components which function as positive or negative modulators of RNA replication.

Several possibilities (which are not mutually exclusive) exist to explain how the defects affecting nsP4 directly would give rise to a host-dependent phenotype. Mutations may alter the structure of nsP4 so that at the nonpermissive temperature it can no longer interact with a necessary host factor which normally functions as a subunit of the viral RNA polymerase. The classic example of this type of host component is the Q β replicase, in which three subunits are derived from the host and one is encoded by the bacteriophage (6). Additional examples of host involvement in viral replication have been observed with animal RNA viruses such as encephalomyocarditis (13), poliovirus (12), and vesicular stomatitis virus (32). As summarized in the introduction, indirect evidence suggests that host components are necessary for Sindbis virus replication in both vertebrate and invertebrate cells. The altered nsP4 encoded by clones 35 and 58 may no longer be able to interact with an invertebrate host cell factor at 34.5°C, resulting in decreased replicase activity. Since these mutants replicated as efficiently as wild-type virus in CEF cells at 34.5°C, the mutant nsP4 must still be capable of functioning at this temperature if certain requirements pertaining to the intracellular environment are met. In this scenario, the temperature sensitivity exhibited by these mutants, in addition to the results from the RNA shift-up experiments, suggests that the enzymatic activity of nsP4 is not temperature sensitive but rather that the formation of an active replication complex is dependent upon the host environment.

A second possibility is that the nonconservative mutations result in the production of only a small fraction of active nsP4. This could result from the mutations being leaky, with the degree of leakiness being affected by the interior cell milieu. Alternatively, the mutations may cause structural changes in nsP4 which render it more susceptible to degradation in mosquito cells at 34.5°C, thus decreasing the level of nsP4 in these cells. Although pulse-chase experiments with wild-type Sindbis virus-infected cells have shown nsP4 to be rather stable at 37°C in CEF cells (21), additional studies suggest that excess nsP4 which is not actively

involved in RNA synthesis may be subject to rapid turnover (29). The stability of wild-type Sindbis virus proteins in mosquito cells has not been studied, and it may be that nsP4 is inherently less stable in invertebrate cells than vertebrate cells. This, coupled with mutations conferring increased susceptibility to mosquito cell proteases at 34.5°C, could result in levels of nsP4 which are insufficient to allow efficient RNA replication.

Limiting amounts of nsP4 might also result from mutations which decrease its rate of synthesis. In Sindbis virus, production of the nsP4 region requires readthrough of an opal termination codon. For both eucaryotes and procaryotes, it has been shown that the nucleotide sequences flanking termination codons can affect readthrough of the codons (15). Specifically, there is evidence which suggests that the nucleotide adjacent to the 3' side of the termination codon plays a role in determining the efficiency of readthrough (14). For Sindbis virus, the first nucleotide following the opal codon is cytosine, a residue which does not usually follow termination codons in eucaryotic organisms (26, 29). It is interesting that this cytosine residue is conserved among several alphaviruses (Fig. 5), even those which do not possess a termination codon at this position. Work with mutant 58.1C1a has shown that a C-to-U change at this position results in significantly lower levels of nsP3 and nsP4 production both in a cell-free translation system (G. Li, unpublished data) and in infected CEF cells (this study).

Virus containing this C-to-U mutation is host restricted and temperature sensitive in mosquito cells. This mutation causes a large reduction in the levels of nsP34 and nsP4 in CEF cells at 34.5 and 40°C, yet the virus grows efficiently in these cells at both temperatures. The most likely explanation is that as with CEF cells, the silent mutation in the 58.1 virus causes a decrease in readthrough efficiency and, therefore, in the rate of nsP4 (or nsP34) production in mosquito cells. In addition, differences in the population of suppressor tRNAs may cause the efficiency of readthrough to vary between cell types; this could result in the context mutation having a more dramatic effect in mosquito cells than in CEF cells. Since readthrough is inefficient in CEF cells, nsP4 is produced in small amounts relative to the other nonstructural

proteins (21, 29). Since the nonstructural proteins are present at lower levels in mosquito cells than in CEF cells, decreased readthrough efficiency may result in an even lower level of the nsP4 domain which is insufficient for viral RNA replication in mosquito cells. This hypothesis is supported by work with two other mutants of Sindbis virus in which the opal termination codon was changed to either an amber or ochre termination codon (29). These mutants, which underproduce nsP34 (and possibly nsP4) and have slight defects in RNA production in CEF cells, also exhibit a temperature-dependent, host-restricted phenotype in mosquito cells. The fact that virus containing the C-to-U mutation can replicate efficiently in mosquito cells at 28°C suggests that temperature sensitivity in these cells might result from decreased readthrough efficiency at the higher temperature. Alternatively, the mutation may decrease readthrough efficiency similarly at both 28 and 34.5°C, but an inherent instability of nsP4 at 34.5°C in mosquito cells may result in levels of nsP4 insufficient for viral RNA replication.

Studies with mutants 58.1, 58.2, and 35.1 provide evidence that the function of nsP4 is critically affected by the cellular environment and strongly suggest that it interacts with or is acted upon by one or more host-derived components which can influence viral replication. Basic differences exist in virus replication in mosquito and vertebrate cells. One striking difference observed in this study was the decreased level of viral protein synthesis in mosquito cells as compared with that in vertebrate cells. For this reason, deleterious mutations which affect the activity or decrease the levels of nsP4 (or other viral proteins) may tend to have more pronounced effects on virus replication in mosquito cells than in vertebrate cells.

ACKNOWLEDGMENTS

We thank W. Reef Hardy and James H. Strauss for antibodies specific for Sindbis virus nsP1 to nsP4. Arash Grakoui for production of the virus stocks used for cDNA cloning, and members of the laboratory for helpful suggestions on the manuscript. We are especially grateful to Guangpu Li for insightful observations on termination codon context effects and for performing the experiment shown in Fig. 3.

This work was supported by Public Health Service grants AI-05920 (V.S.) and AI-24134 (C.M.R.) from the National Institutes of Health and by a grant from the Pew Memorial Trust. J.A.L. was supported in part by Public Health Service Infectious Disease/Microbiology Training grant AI-07172 from the National Institute of Allergy and Infectious Diseases. C.M.R. is a Pew Scholar in the Biomedical Sciences.

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